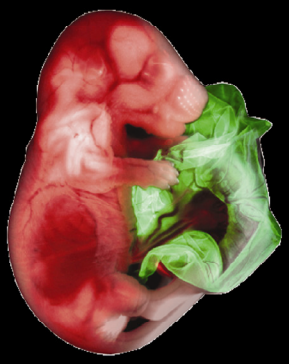
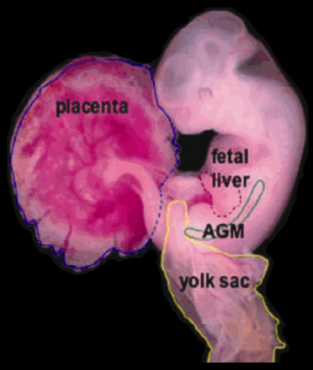


The Origin and Future of Hematopoietic Stem Cells



Kimberley Kolijn

April 2012

Explanation Cover

On the cover are two mouse embryos: one is a 10.5 day old mouse embryo (right) and the other is 18.5 day old embryo with yolk sac (left, GFP labeled) and the placenta (red color starting from the 'navel') (By courtesy of Bettina Bambacht).

References

Double transgenic mouse embryo, 18.5 days (17x), Brightfield, Darkfield, Fluorescence (GFP and RFP). / Gloria Kwon, Memorial Sloan-Kettering Insititute.

Mikkola, H.K., Fujiwara, Y., Schlaeger, T.M., Traver, D., Orkin, S.H., Blood, 2003, Expression of CD41 marks the initiation of definitive hematopoiesis in the mouse embryo.

Table of Contents

Abstract

Hematopoietic stem cell (HSCs) therapy in the form of bone marrow transplantations has been used successfully in the clinic for over 40 years and continues to save lives daily. Clinical stem cell transplantations are required to reconstitute the hematopoietic system of cancer patients that have undergone chemotherapy and/or irradiation. Nevertheless, there are still many obstacles with the clinical use of HSCs, including limited availability of transplantable HSCs, donor matching and graft versus host reaction and the difficulty to expand HSCs *in vitro*. Embryonic stem cells (ESCs) and/or induced pluripotent stem cells (iPSCs) could offer a solution to this problem by providing a means to generate HSCs *in vitro*. The knowledge to achieve this will likely come from our understanding of the origin of HSCs in the embryo. In this review, I will discuss the ontogeny of HSCs and the prospects of using ESCs and/or iPSCs to generate HSCs.

Chapter

1. Hematopoietic Stem Cells in Adults

During the entire life of an individual, blood cells circulate to deliver nutrients, oxygen and to provide immune protection. The lymphoid cells (T-, B-, and natural killer (NK) cells) and cells from the myeloid lineage such as neutrophils, eosinophils, basophils, monocytes, and macrophages protect organisms from pathogens. Megakaryocytes and platelets play an important role in the coagulation process and erythrocytes in oxygen delivery to tissues.

All blood cell types are produced from hematopoietic stem cells (HSCs) through a process known as hematopoiesis (Figure 1). By constantly producing mature blood cells, HSCs maintain blood cell homeostasis throughout the life of an organism. Production of blood cells reaches high levels; erythrocytes are generated at a rate of 1 to 5 million cells per second and each cell circulates the blood system for approximately 3 months.

HSCs are characterized by two main properties; multipotency that allows the cells to proliferate and differentiate, and self-renewal capacity which implies that during mitosis the cells produce at least one cell identical to them. Self-renewal capacity is a property of stem cells and is limited in progenitors and late progenitors (Figure 1). Progenitors are multipotent and can generate cells of different lineages (lymphoid, myeloid and/or erythroid), whereas late progenitors are unipotent and only give rise to one specific cells type (e.g. a pro-E cell only gives rise to erythrocytes). To maintain the number of blood cells constant throughout life only few HSCs divide, while progenitors proliferate extensively. This reduces the risk of DNA and cell damage of HSCs that could cause the frequency of replication to gradually decline and eventually stop (replicative senescence).

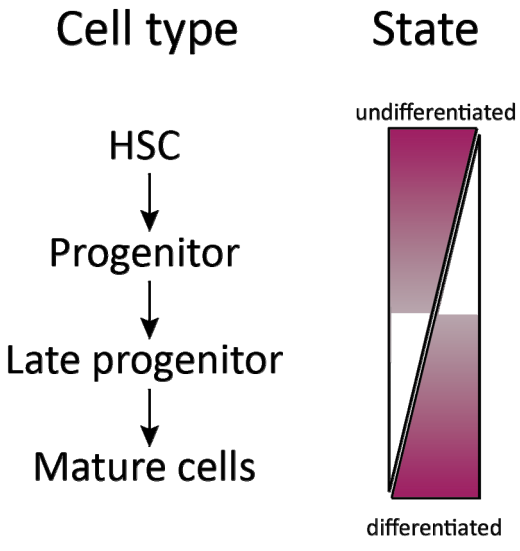


Figure 1. Hierarchy of hematopoietic cells

Hematopoietic stem cells (HSCs) give rise to differentiated mature cells that constitute the blood system. This is established through the proliferation of intermediary progenitors and late progenitors to reduce the risk of DNA and cell damage to the HSCs.

In the 1960s, Till and McCulloch injected bone marrow (BM) cells into irradiated adult mice to identify cells that allow the regeneration of a new blood system [1]. They found that the injected BM cells formed colonies in the spleen (colony forming units or CFU) and differentiated into different blood cell lineages to reconstitute the blood system in these animals. Unintentionally, they identified the first stem cells and laid down the foundation for all current stem cell research. Studies in the BM and serial transplantations however showed that the self-renewal ability of these CFU-S (CFU in spleen) is very limited [2-4]. Further experiments with single, retrovirus labeled, BM cells done in the late 1980s, nevertheless demonstrated that a single cell could in fact generate all blood cell types in mice for at least six months [5-7]. Upon secondary engraftment the same viral integration was found in all blood cell lineages of the recipients, thereby displaying the self-renewal and clonal expansion abilities of a single cell.

Thus far, HSCs are the only stem cells that have gained enough understanding to use them in the clinic effectively. HSCs are the key element in transplantation protocols and can be isolated from BM, mobilized peripheral blood, or umbilical cord blood. Stem cells isolated from bone marrow or blood need to be human leukocyte antigen (HLA) match to avoid rejection in a graft-versus-host reaction. Therefore, many patients in need of donor cells cannot use cells extracted from bone marrow. Stem cells in human umbilical cord however, require less stringent HLA matching and can be more readily obtained. The use of umbilical cord blood as a source of HSCs has therefore steadily grown. However, as the number of HSCs in umbilical cord blood is limited, the use of BM HSCs cells is still the predominant form of transplantation.
BM transplantations were the first successful cell replacement therapies in patients and they have been a standard approach for the treatment of many hematological cancers and disorders (e.g. myelomas, primary immunodeficiency, aplastic anemias and leukemia) for over 50 years. HSCs therapy has been most successful as a treatment, though scientists have so far been unable to maintain them *in vitro*. Furthermore, the isolation procedures for HSCs are far from efficient as currently only heterogeneous cell populations of stem cells and progenitors can be obtained [8-11]. To use HSCs in the clinic optimally, their biology and ontogeny needs to be further studied.

In the mammalian adult, HSCs reside in the red part of the BM near the sinusoids (small blood vessels) and near osteoblasts in the connective tissue lining the medullary cavity of a bone (the endosteum) [12-14]. This microenvironment, or niche, affects HSC functions such as self-renewal, apoptosis/survival, differentiation/proliferation and cell cycle progression. As the amount of HSCs in the BM is very low, these functions need to be tightly regulated. The endosteal and vascular niche

regulate HSCs through complex interactions between different cell types like mesenchymal stromal cells, osteoblasts, endothelial cells, and adipocytes. Additionally, extracellular factors such as the extracellular matrix, growth factors, cytokines, adhesion molecules and the physiochemical nature of the niche are also involved in HSC regulation. Next, I will discuss some of the important factors in the adult HSC niche.

1.1 Bone Marrow Niches and HSC Markers in Adults

Many adhesion molecules, transcription factors, cell cycle regulators and signaling pathways are involved in the interactions between the niche and HSCs. Signaling pathways that have been well studied in the adult BM include Notch, bone morphogenetic protein (BMP), Wnt, transforming growth factor β (TGF-β) and hedgehog (HH) [14-18]. C-KIT, Tie2/Ang-1 and the Ca2+ sensing receptor also play a role in HSC regulation [19-21].

The Notch pathway is highly conserved and controls many different cell differentiation processes. It has been reported by several groups that binding of the Jagged-1 ligand to the Notch1 receptor inhibits the differentiation of HSCs and stimulates their self-renewal [15, 22, 23]. BMP and Notch are important for the regulation of the HSC BM niche and osteoblastic cells play a key role in this regulation [14, 15]. The Wnt pathway is involved in the self-renewal of HSCs demonstrated by the overexpression of activated β-catenin that leads to an expansion of HSCs *in vitro* [16, 23]. The c-KIT receptor in particular is crucial for HSC self-renewal [19]. Stem cell factor (SCF) signaling through c-KIT promotes the proliferation and survival of HSCs and their release from the niche. The tyrosine kinase receptor Tie2 is expressed on HSCs and endothelial cells. The Tie2 ligand angiopoietin-1 (Ang-1) is expressed by osteoblasts and binding to Tie2 leads to HSC quiescence [20].

One important reason to study HSC regulatory pathways is to eventually purify HSCs from tissue with the use of cell surface markers. Many surface proteins have been identified and can differ between mice and men, but so far it has been possible to purify adult type HSCs to 50% homogeneity using flow cytometry in mouse. Complete homogeneity has not yet been achieved, due to expression of these markers on other progenitors. Some general histological, not HSC specific, stainings such as BrdU (HSC retain the label), Hoechst (HSCs efflux the dye and are in the quiescent fraction) or stainings for metabolic activity, like Rhodamine 123, can also be used.

An important combination of markers for HSCs is the absence of lineage commitment antigens (lin-) such as GR1 and MAC1 (neutrophils and monocytes), CD3, CD4 and CD8 (T-cells), CD19 or B220 (human and mouse B-cells respectively), and Ter-119 (erythrocytes) that are normally expressed on the surface of mature blood cells and absent on undifferentiated stem cells [24]. Low expression of the glycoprotein Thy1.1 (Thy1.1^{lo}) and the expression of the Sca-1 antigen (Sca-1^{+}) on HSCs are also used

to isolate HSCs [10, 25, 26]. However, only 50% of HSCs are stained for Sca-1, due to the properties of the Sca-1 antibody. Recently, there has been progress in the visualization of HSCs when Ma et al. showed that the Sca-1 transgene can visualize HSCs and some hematopoietic cells [27]. The transgene expresses GFP under the control of the Sca-1 (Ly6A) promotor after which GFP labeled cells can be followed *in vivo* [27, 28].

Subsequent studies have shown that the fraction of Lin$^{-/lo}$/Thy1.1^{lo}/Sca-1+ BM cells still contain cells with diverse self-renewal activities and can be further fractioned based on expression of the c-KIT receptor [29]. The c-KIT receptor is expressed in HSCs and controls the activation of HSCs and their subsequent release from the niche [19]. C-KIT is part of a combination of markers that is frequently used to enrich for adult HSCs, the Lin-/Sca1+/c-KIT^{hi} or LSK population. Within the LSK population, the cell surface sialomucin CD34 is expressed in HSCs that can reconstitute the hematopoietic system of irradiated animals for short term (ST-HSCs), but not for long term (LT-HSCs) [7, 30].
Another important group of adult HSC markers include the SLAM-family members CD150, and the negative expression of CD48, CD244, and CD34 [13]. Currently, a BM LT-HSC has the following phenotype Lin-/Thy1.1^{lo}/Sca-1+/c-KIT^{hi}/CD34$^{-/lo}$/CD150+/CD48-/CD244-/CD38+ and/or Flk2- and a ST-HSC Lin-/Thy1.1^{lo}/Sca-1+/c-KIT^{hi}/CD34+/CD150+/CD48-/CD244-/CD38+ and/or Flk2+ [13, 27, 30, 31]. It is clear that there is no unique HSC marker and that HSCs can currently only be isolated with a combination of markers. At present, true HSCs can only be identified based on their functional properties (e.g. self-renewal and pluripotency) in an *in vivo* transplantation assay.

2. Hematopoiesis in the embryo

To expand the use of HSCs beyond their current applications and to generate or maintain them *in vitro*, we need to understand how they arise and function. As the adult stem cell compartment is established during embryonic development, research has concentrated on the (molecular) mechanisms behind stem cell generation in the embryo. In the embryo, HSCs are present in different anatomical locations at different time points during development. By midgestation, the major hematopoietic organ, the fetal liver (FL), and the thymus have been colonized by extrinsic progenitors and produce hematopoietic cells [32]. Before colonization of the FL, HSCs can be found in the yolk sac (YS), the aorta-gonad-mesonephros (AGM) region, the major arteries and the placenta. Their cellular origin is however unclear, as the onset of circulation makes it nearly impossible to assess the original site of generation. Here I will discuss the ontogeny of the hematopoietic system followed by the current state on HSC generation through stem cells differentiation or dedifferentiation of mature cells.

Hematopoiesis in the embryo occurs in two distinct successive waves: an embryonic ('primitive') wave and a definitive ('adult') wave (Figure 4). The first (primitive) wave is primarily erythropoiesis (production of erythrocytes) and the production of transient progenitors. Erythroid progenitors are present from embryonic day (E) 7-8.5 of mouse development and E15 of human development. They generate nucleated, immature erythrocytes expressing embryonic globins instead of the β-major and α globins expressed in adult [33]. Macrophages generated during the primitive wave lack lysozymes and peroxidase activity which allows them to bypass the monocyte stage for an accelerated maturation [34, 35]. These primitive blood cells are the first cells to circulate and cover the early basic needs of the embryo (e.g. erythrocytes for proper oxygenation of the embryo and macrophages and megakaryocytes for tissue remodeling and cell damage restoration).

2.1 The Yolk Sac

The first primitive erythrocytes in mammals are produced in the extra-embryonic YS from homogeneous cellular aggregates in the mesodermal layer referred as blood islands (Figure 2A and B) [36-39]. The YS is an extra-embryonic membranous sac and is connected to the midgut by the yolk duct, through which nutrients are transported to the embryonic circulation. The outer rim of the blood islands acquires an endothelial identity and the core cells differentiate into erythrocytes and form the lumen of the blood islands [38].

In the 1970s, the hypothesis prevailed that HSCs originate in the YS. This statement was reinforced by several experimental lines of evidence. The removal of the yolk sac in E7 mouse embryos resulted in

a failure to develop hematopoiesis in the FL [40]. Similar studies with YS cells injected into mouse embryos of related stages indicated that cells of donor origin were present in the thymus and BM of injected mice [41, 42]. Another group demonstrated that cells isolated between E8-9 from the mouse YS had T cell (lymphoid) differentiation potential, a feature attributed to HSCs [43]. The first *in vitro* colony assays done by Moore and Metcalf with cells from the yolk sac and the embryo proper in mice, showed that only cells from the yolk sac developed hematopoietic activity after two days in an explant culture [36, 44]. One group however, found that reconstitution of the B cell compartment of adult irradiated mice occurred with a similar or higher efficiency with cells from the intraembryonic compartment than those from the YS [45]. This experiment raised the possibility that the intraembryonic compartment could contain lymphocyte progenitors before colonization of the FL, though these findings were not addressed until the late 1980s.

2.2 The Aorta-Gonad-Mesonephros Region

Around the late 1980s, avian grafting experiments done before the emergence of blood cells and the onset of circulation, conclusively demonstrated that definitive hematopoiesis originates from a mesodermal area surrounding the dorsal aorta and not from the YS (Figure 2A and C) [46, 47]. Chimeras of a quail embryo and a chick YS constructed before the onset of circulation (to prevent cross-contamination by circulating progenitors), were followed for their contribution to hematopoiesis up to the hatching process. Quail and chick cells are easily discernable with the use of species-specific antibodies or visually by their nucleoli; nucleoli in chick are dispersed and in quail condensed [48, 49]. This particular grafting experiment showed that the contribution of the YS to hematopoietic cells is substantial in the early developmental stages and absent at the later developmental stages. Subsequent experiments demonstrated that adult B and T lymphoid cells arise solely from the intraembryonic compartment [50, 51]. The group of Lasilla established that the origin of HSCs lies within the intraembryonic compartment and that YS progenitors cannot sustain adult hematopoiesis or give rise to lymphoid cells.

Evidence in mammals was later obtained, when progenitors with B lymphoid potential were found in the intraembryonic compartment of mouse embryos [52, 53]. Further research narrowed the area of progenitors to a region that encompasses the aorta, gonads, and mesonephros (AGM region) [54, 55]. The AGM region arises from the para-aortic splanchnopleura (P-Sp), a cell layer which is formed during development. It consists of a layer of lateral plate mesoderm and endoderm at the pre-somite stage (E7.5-8 in mouse, E19-23 in human embryo) (Figure 2A) [37, 56, 57]. The P-Sp develops into the endoderm of the developing gut, the omphalomesenteric artery, the dorsal aorta and their

splanchnopleural linings (E8.5-10 in mouse, E 25-30 in human) [37, 58]. Around E10-11.5 in the mouse and E30-40 in humans, when colonization of the FL has begun, it also includes the gonads and mesonephros (the AGM region) [58].

The P-Sp produces hematopoietic progenitors around the pre-somite stage (E8.5 in mice embryos) [56, 58, 59]. As this is after the connection of YS and intraembryonic blood vessels, the presence of hematopoietic progenitors could still be the result of either *in situ* generation or the aggregation of cells generated elsewhere. This issue could not be adressed with previous techniques as the embryo's circulation enables the migration of cells from one site to another. However, this problem was circumvented by an organ culture technique that allows the culture of an organ dissected before circulation, outside of the embryo. With this technique, the YS and P-Sp were isolated before the connection of the vessels, allowed to develop *in vitro* and analyzed for hematopoietic potential [56]. The P-Sp explants contained multiple lineages, whereas the YS only generated erythro-myeloid cells.

The AGM harbors the first HSC from E10.5 (Figure 4). Intra-embryonic hematopoietic progenitors isolated from the P-Sp before E10.5 were not considered HSCs due to the lack of long term reconstitution (LTR) upon transplantation into irradiated recipients [41, 56, 60, 61]. Yoder et al. could merely uncover LTR activity from P-Sp progenitors extracted at E9 upon direct injection into the liver of newborn immunodeficient mice [62]. The absence of LTR activity of early intra-embryonic progenitors was thought to be the result of low level expression of MHC class I molecules rendering a cell susceptible for elimination of the hosts radiation-resistant NK cells [63]. Concomitantly, transplantations into transgenic *Rag2*$^{-/-}$, γ*c*$^{-/-}$mice, which are alymphoid and lack NK cells, showed interesting results [64] Indeed, these severe immunodeficient mice showed a higher level of engraftment of transplanted cells. Moreover, cells isolated from the P-Sp before E8.5 and grown in organ culture for several days before transplantation, displayed LTR (up to 8 months after transplantation), in contrast to the short term reconstitution (STR) of YS derived cells [59].

Cells from the P-Sp cultured under stringent limiting-dilution conditions have established that generation of hematopoietic progenitors in the P-Sp/AGM starts from E8.5, peaks around E10.5, and is diminished by E13 [58]. LTR activity in the YS and FL can be seen one day later than in the AGM, which suggest they are seeded by AGM-derived HSCs [61, 65]. In conclusion, results suggest that the YS seems primarily involved in primitive hematopoiesis and the AGM in definitive hematopoiesis.

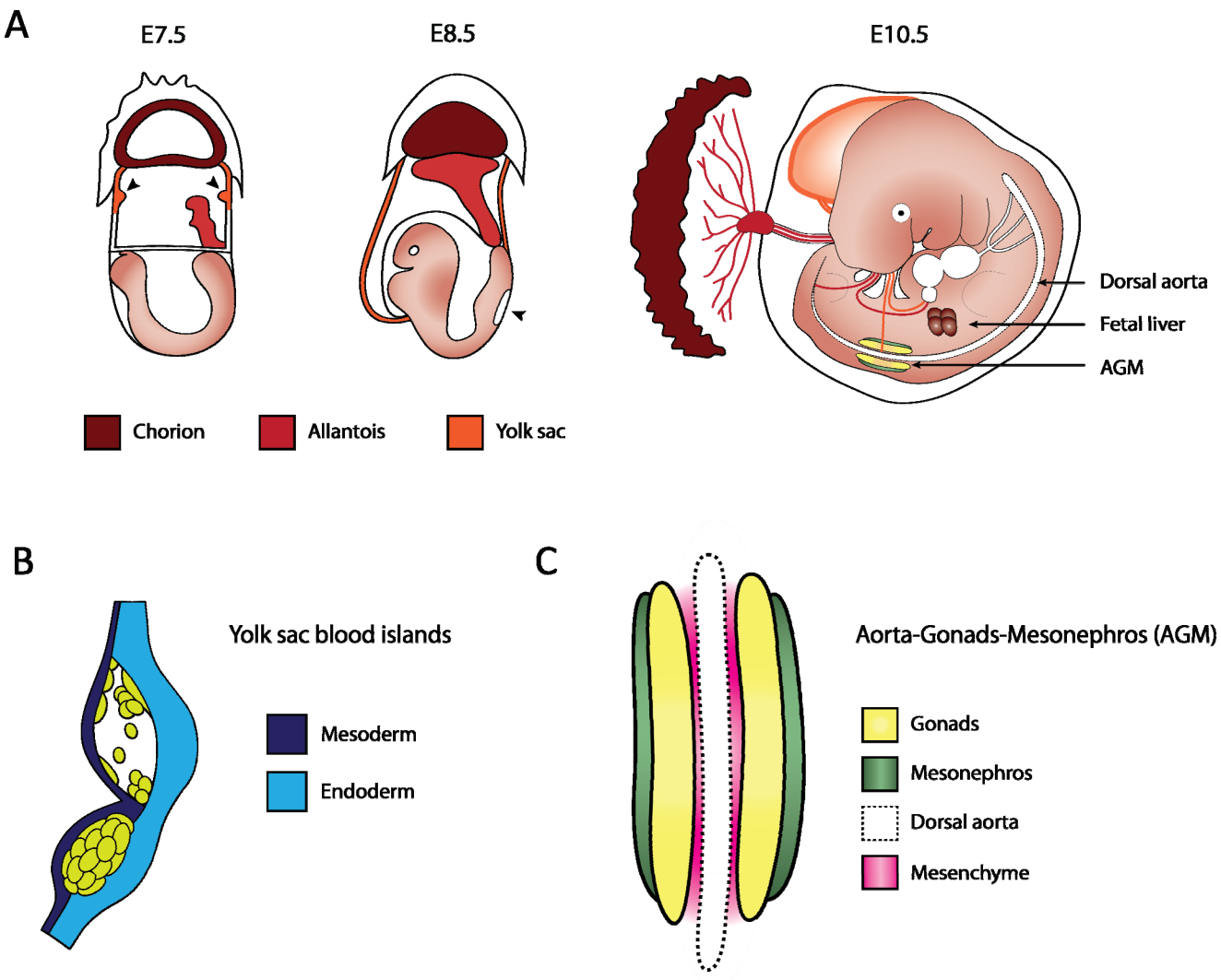


Figure 2. Sites of HSC generation in the mouse embryo

Hematopoietic stem cells (HSCs) are first generated in the yolk sac around embryonic day (E) 7.5 in mice embryos (panel A, arrowheads and panel B).The blood islands form between the endodermal and mesodermal layers (panel B). At E8.5, the chorion and allantois have joined to form the placenta and the P-Sp (panel A, arrowhead) is present. The AGM produces the first adult definitive HSCs (panel A and C) around E10.5, which migrate to the fetal liver and eventually the bone marrow before birth.

2.3 Location of HSCs within the AGM

The AGM is a large region that comprises the dorsal aorta, genital ridges and mesonephros (Figure 3). Researchers have been interested to determine the exact location of HSC generation within the AGM. The first experimental data in avian models suggested the dorsal aorta and surrounding tissues as sites of HSC generation[66]. If a quail aorta was grafted into the dorsal mesentery of a chick embryo, they found that the HSCs originated from the quail aorta. However, upon removal of the surrounding mesenchymal cells, the quail aorta showed no contribution to hematopoietic cells [67]. This led the authors to believe that in chick, the tissue surrounding the dorsal aorta was the contributing factor to HSC generation. In mice however, Godin et al. found 55% of the HSCs in the AGM to be concentrated in the aorta (55%) [68]. Dzierzak's group injected cell suspensions of the dorsal aorta with its surrounding mesenchyme and urogenital ridge segments into adult irradiated mice and found the highest frequency of LTR in mice injected with cells from the dorsal aorta [69].

In the dorsal aorta, two structures seem important in HSC generation. The first structure is present within the ventral wall of the dorsal aorta and is referred to as intra-aortic hematopoietic clusters (IAHCs) in both birds and mice (Figure 3B) [70, 71]. Avian chimera cell-fate map experiments have shown that the dorsal and ventral aorta have different embryonic origins, that could explain the presence of clusters exclusively in the ventral aorta [72]. In mice however, the dorsal wall of the aorta also contains these clusters of hematopoietic cells as well as the omphalomesenteric/vitelline and umbilical arteries [69, 73-75].

During development, the number of IAHCs peaks at the same time as the generation of progenitors in the AGM, E10.5, and IAHCs are present in the large vessels of the embryo and in the YS. Although IAHCs outside of the aorta are not well characterized, they are known to harbor HSCs with LTR activity.

The second structure important in HSC generation is sub-aortic patches (SAPs), which are present in the mesenchymal cell layer below the aortic floor (Figure 3B). They were first identified through the expression of the GATA-3 transcription factor and preferentially localize below IAHCs [76, 77]. SAPs can be detected during HSC generation and disappear around the time the AGM no longer produces progenitors [68, 76].

Unfortunately, the functions of both IAHCs and SAPs are not fully understood. Both structures have been identified in human embryos, though direct evidence of their involvement in the generation of HSCs has only been demonstrated in *Xenopus* embryo's [78-80].

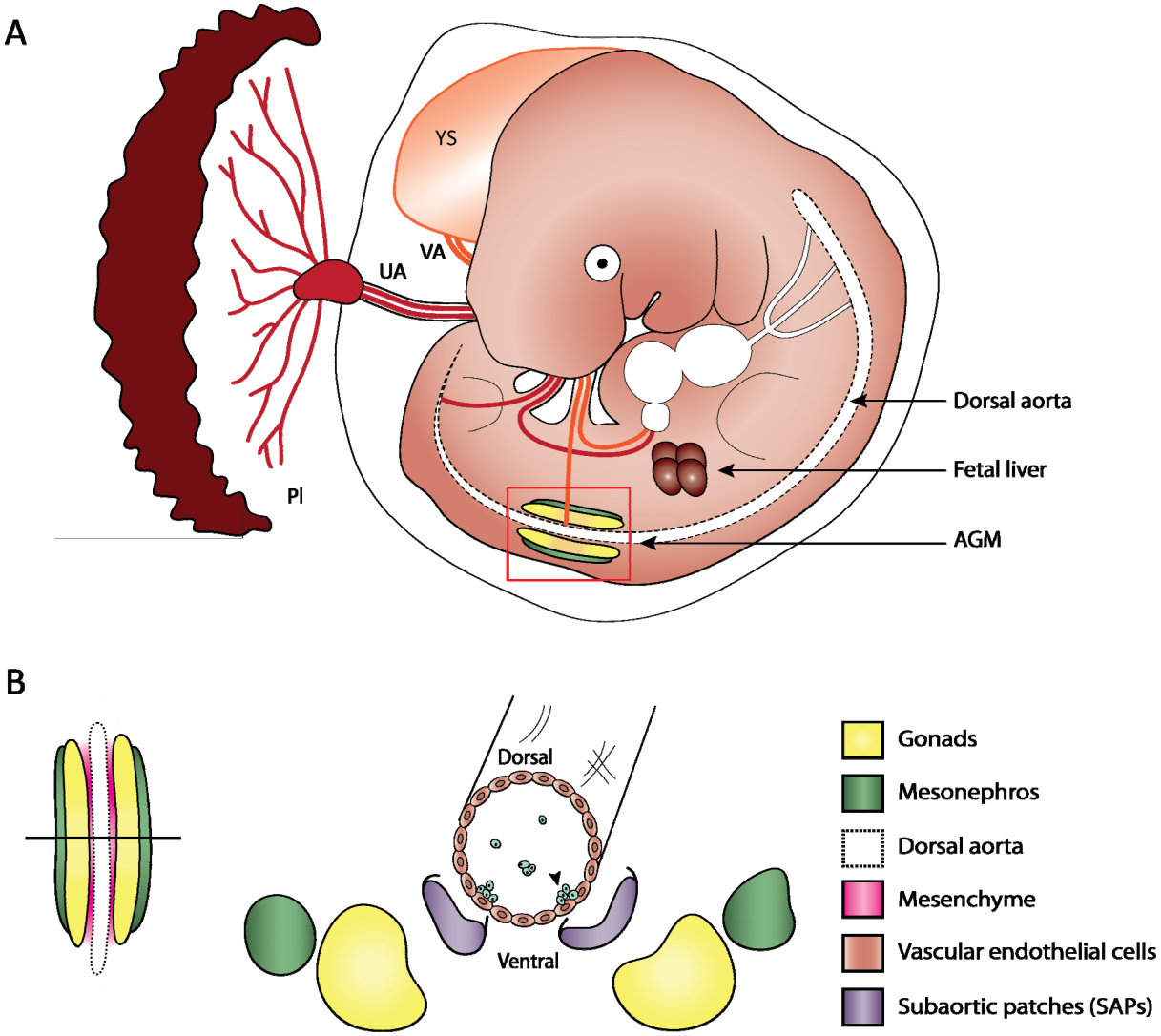


Figure 3. The mouse AGM region

Panel A illustrates an overview of hematopoietic sites in the mouse embryo. The placenta (Pl) is connected to the embryo with the umbilical arteries (UA). The vitelline arteries (VA) supply the embryo with nourishment from the yolk sac (YS). The aorta-gonad-mesonephros (AGM) region in panel B contains sub-aortic patches (SAPs) and intra-aortic hematopoietic clusters (IAHCs, arrowhead) that are important in the formation of definitive HSCs.

2.4 The Hemogenic Endothelium

The presence of IAHCs in the embryo indicates a close association of HSCs with the endothelium. Around 1910, it was already postulated that a common mesodermal precursor – a hemangioblast – could produce both endothelial cells and hematopoietic cells. The 'hemogenic-endothelium' hypothesis arose when initial evidence in the yolk sac supported the existence of hemangioblasts that contributed to the blood islands [81, 82]. Later experiments also indicated an important relation between endothelial and hematopoietic cells [83-86]. Eichmann *et al*. discovered that mesodermal cells could give rise to either a hematopoietic cell colony in the absence of vascular endothelial growth factor (VEGF) ligand or to an endothelial cell colony in the presence of VEGF ligand [87].

It is suggested that hematopoietic progenitors either transdifferentiate from mature vascular cells or are produced locally from hemangioblasts. It is also possible that the mesenchyme under the aortic floor (SAPs) gives rise to HSCs after which they migrate towards the lumen and are released into the blood stream [88]. Observations have strongly supported the hemogenic endothelial hypothesis as the labeling of all endothelial cell with low-density lipoproteins (LDL) before the presence of any clusters, leads to labeling of IAHCs one day later [88-90]. Additionally, hematopoietic cells could be recovered from progenitors isolated through the endothelial markers vascular endothelial cadherin and TIE2/TEK. Mutations in Flk1 and TIE2 seem to affect the migration and formation of endothelial and hematopoietic cells, which suggests a lineage relationship between the two cell types [91, 92]. Moreover, researchers were able to obtain both endothelial and hematopoietic cells *in vitro* from single progenitors [93-96]. North *et al.* were also able to obtain HSCs from mice transplanted with endothelial cells isolated at E11.5 [97]. Recent bioimaging approaches have now proven that endothelial cells can give rise to hematopoietic cells at the single cell level and that a hemogenic endothelium truly exist [98-100].

2.5 Embryonic Sites of HSC Generation

Until now, only one site has unambiguously been identified as a site of definitive autonomous HSC generation, maintenance, and expansion; the P-Sp/AGM ventral aorta. [61]. This has however established a dogma in which the intra-embryonic HSCs are the main source of definitive HSCs, even though very low numbers of definitive HSCs are produced during a very short time in the AGM and the major arteries [56, 60, 61, 68, 69]. Therefore, it was questioned if the HSCs from these regions could be the sole contributor to the large amount of HSCs colonizing the liver around E11. The FL is incapable of *de novo* generation of hematopoietic progenitors, but taking in the large HSC pool in the FL and the length of the cell cycle, a substantial number of HSCs is likely generated at other unknown sites [101, 102].

Recent studies have revealed that the placenta harbors a major pool of HSCs (>15 fold more HSCs than in the AGM) (Figure 2A) [103]. The placenta is a highly vascularized tissue produced by the embryo during pregnancy, which provides an interaction between the mother and the fetus without direct contact of their bloodstreams. It was already discovered in the 60s that the fetal placenta contains HSCs, though the general assumption was that the placenta was purely colonized by HSCs from the FL [1, 104]. At that time the placenta was thought to be solely involved in immune protection, production of hormones, and exchange of nutrients and wastes between the mother and fetus. Renewed interest in the placenta as a hematopoietic organ came when studies showed that the avian allantois has hematopoietic activity [105]. Subsequently, Alvarez-Silva *et al*., showed that the mouse placenta also harbors a substantial number of multi-potential clonogenic progenitors before the onset of liver colonization [106]. *In viv*o studies of the midgestation mouse placenta confirmed the presence of a large pool of HSCs from E11 with the capacity to self-renew and provide hematopoietic cells from all lineages [103, 107].

The murine placenta contains a maternal part and a fetal part derived from polar trophoectoderm and allantoic mesoderm. The allantois fuses with the chorionic plate around E8.5 and gives rise to the fetal placenta and umbilical vessels with their associated stroma (allantois) and the three trophoblast cell layers (chorion) (Figure 2A) [108]. Definitive HSCs appear in the AGM by E10.5-11, concomitantly with the formation of the AGM region (Figure 4) [103, 107]. By E12.5, the labyrinth facilitates the exchange of oxygen between maternal and fetal red blood cells, and the definitive HSC pool consists of approximately 13-50 HSCs. The number of HSCs in the placenta declines by E15.5, which likely reflects their migration to the FL [103, 107].

Two groups separately demonstrated that the allantois and the chorion can independently generate

HSCs *de novo* when dissected before circulation or in the absence of circulation [109, 110]. A sodiumcalcium exchanger (Ncx1)-null mouse which fails to initiate a heartbeat at E8.5, and thus has no circulation, showed that hematopoietic clusters similar to IAHCs still form and produce lymphoid and myeloid cells upon explant culture [109]. However, though Robin *et al*. could not confirm the autonomous generation of definitive HSCs of the wild type placenta explant cultures, they identified the human placenta as a HSC niche [111, 112]. The question that remains why the placenta is required for HSC generation as the placenta is a typical mammalian structure and hematopoiesis develops normally in non-vertebrates.

Even though several groups have now demonstrated that HSCs are generated *de novo* in the AGM, some experiments find only a contribution of yolk sac cells to definitive HSCs. One study used a non-invasive Cre/loxP recombination labeling system at E7.5 to trace all Runx1 positive cells *in vivo* [113]. With this technique they stained only yolk sac Runx1 positive cells and show that these cells develop into adult HSCs that colonize the umbilical cord, the AGM and FL. In contrast, Yoshimoto *et al*. showed that both AGM and YS have autonomous B-lymphoid potential that initiates from hemogenic endothelium at E9 in Ncx1-/- null mutant mice [114]. One group claims that all cells that seed the FL are derived from the YS, as Ncx1 knockout mice that lack a heartbeat, have a normal number of definitive erythroblasts and hematopoietic progenitors in the YS and virtually none in the embryo proper [115]. However, it was recently discovered that biomechanical force applied to vascular walls by fluid shear stress activates the nitric oxide signaling pathway and increases the expression of hematopoietic markers such as Runx1 in endothelial cells [116, 117]. In the absence of shear stress only few HSCs develop, indicating that the mechanical force of the bloodstream is required for the development of definitive HSCs. Additionally, they show that in Ncx1 mutant mice, the vascular endothelium develops normal and that defects in the mutant tissues could be overcome by subjecting them to shear stress. Therefore it seems likely that the YS is not the sole contributor to definitive HSCs.

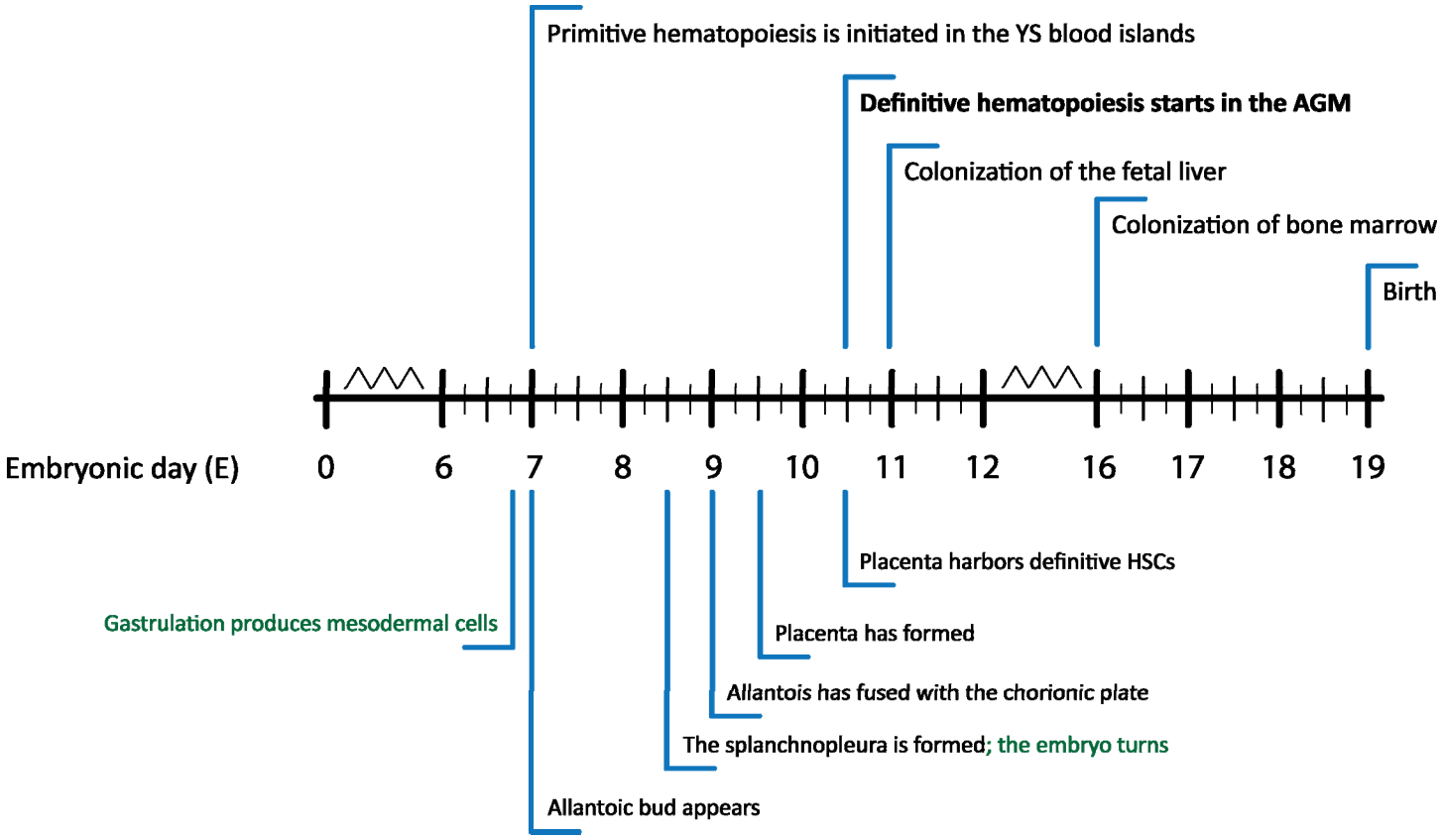


Figure 4. Hematopoietic time line of the mouse embryo

The yolk sac (YS) produces the first hematopoietic cells around E7. The chorion and allantois start forming the placenta and around E10.5 the AGM generates the first definitive HSCs. Colonization of the liver follows shortly after and before birth, all HSCs have migrated to the bone marrow.

2.6 HSC Niche and Markers in the Embryo

Embryonic HSCs and progenitors express some cell markers that are different from adult HSCs in the BM, but there are also many similarities. Some genes are expressed, but in a time dependent manner, whereas others persist throughout development. One of the first markers to be identified was AA4.1 and it can be found on the majority of HSCs [58, 118, 119]. Later in development, AA4.1 is present on lymphoid progenitors throughout the embryo [120]. Sanchez et al. was the first group to show that LTR cells in the AGM, were all present in the c-KIT+ fraction and expressed low levels of MAC-1/CD11b [121]. Mutations in the proto-oncogene c-KIT receptor and it's ligand c-KIT do not cause severe defects to definitive hematopoiesis but disrupt definitive erythropoiesis and FL hematopoiesis [122, 123].

Together with c-KIT, CD41 is the first marker to be expressed in the yolk though compared to the AGM c-KIT+ fraction, the YS has less lymphoid progenitors than the AGM [119, 124, 125]. Expression of the hematopoietic marker CD41 and vascular endothelial cadherin (VE-cad) in the absence of CD45 has been ascribed to pre-HSCs which can mature into LT-HSCs, though pre-HSCs are also present in the VE-cad+/CD45+ fraction with or without expression of CD41 [119, 124, 126-128]. Robin et al. recently showed that HSCs are predominantly present in cells that express intermediate levels CD41 until E12 when AGM HSC preside in the CD41- fraction [129]. They speculate that CD41 marks HSCs in the aorta that transit from endothelial to hematopoietic fate and lose their expression of CD41 during the transition. This also explains why previous experiments have identified HSCs in both CD41- and CD41+ fraction.

The cell surface glycoprotein CD34 and CD38 are also expressed in a similar time dependent manner [130, 131]. In the 90s, Sanchez et al. found that c-KIT positive HSCs co-express CD34+ [121]. Later on, LT-HSCs were found enriched in the Lin-/c-KIT+/CD34+ fraction, but in mice older than ten weeks this enrichment shifted towards the Lin-/c-KIT+/CD34- fraction. Hematopoietic progenitor cells, unlike HSCs, express CD34 throughout murine development. CD38 expression however only seems to be expressed in newborn HSCs and expression is lost around the 5th week [131].

Another glycoprotein referred to as CD31/PECAM-1, is also expressed in endothelial cells together with CD34 and marks hematopoietic cells with LTR activity [119, 132, 133]. McKinney et al. recently found that the earliest embryonic HSCs are CD41+/CD34+/CD45-/CD150- and that mature HSCs have the exact opposite phenotype (CD41-/CD34-/CD45+/CD150+) [128].

In the embryo, the Ly6A-GFP construct can visualize or enrich the fraction of HSC isolated from tissues, just like in the BM. The construct marks all HSCs in the embryo and expression can be seen in the dorsal aorta, urogenital regions and FL at E11 [28]. In the embryo, green fluorescent protein (GFP) expression in Ly6A-GFP mice is present in some c-KIT+ cells and even though Sca-1 is only expressed at low levels in the embryo around E10.5-E11, cell-sorting experiments have shown an enrichment for LT-HSCs in the Sca-1+ fraction [28, 118]. Indeed, transplantation studies have confirmed that the Ly-6A GFP construct labels all embryonic and adult HSCs [28]. The fact that Sca-1 is also expressed in mesenchymal and endothelial cells is another indication of the close relationship between endothelial and hematopoietic cells [27, 28, 103].

In situ stainings have shown that progenitors during primitive hematopoiesis express several factors such as Lmo-2, Tal-1/SCL, GATA1, GATA2, and Runx1. The T cell oncoprotein Lmo2 is thought to maintain erythroid progenitors in an immature state and promote self-renewal which is in concurrence with the finding that null mutants do not survive after E10.5 due to a failure in YS erythropoiesis [134]. However, Lmo2 is also required for definitive hematopoiesis as in null mutants no contribution to adult hematopoietic cells is detected [135]. Lmo2 is the bridging factor in a large transcription complexes in which it binds to Tal-1/SCL and GATA1 [136]. Tal-1/SCL and GATA1 have also separately been shown to be involved in hematopoiesis. Without the T-cell leukemia oncoprotein Tal-1/SCL no hematopoietic cells can be detected and inactivation of the transcription factor GATA1 leads to a failure in the differentiation of pro-erythroblasts to erythrocytes in primitive hematopoiesis [137, 138]. Besides GATA1, Lmo2 can also bind to GATA2 and GATA3 [139]. GATA2 seems to play an important role in definitive hematopoiesis, while GATA3 is expressed in the IAHCs and SAPs and is involved in the generation of T-cells [76, 140].

There are some mutations that affect only definitive hematopoiesis such as the previously mentioned AML1/Runx1 protein. AML1/Runx1 encodes the DNA-binding subunit of a transcription factor which is expressed preferentially in IAHCs as well as in the underlying mesenchyme [141, 142]. It is required for the differentiation of definitive hematopoietic cells in the entire embryo and marks LT-HSCs [97, 143]. Runx1 is required for the expression of human CD34 in LT-HSCs [144]. Runx1-/- mouse embryos establish normal primitive hematopoiesis but die around E12 because HSCs and definitive hematopoiesis are absent [97, 141, 145-147]. IAHCs are also no longer present, which implies that Runx1 could be involved in the transition of endothelial cells to hematopoietic cells. Runx1+/- haplosufficient embryos are very interesting because though they produce only few numbers of HSC, they survive the mutation and can be used to study definitive hematopoiesis [111, 148].

Recently, the transcription factor Sox17 was found to be specifically expressed in fetal HSCs. Ectopic expression in adult progenitors converts them to self-renewing HSCs with fetal characteristics [149]. Moreover, besides apparent dedifferentiation of adult hematopoietic cells, Sox17-/- embryos do not generate definitive HSCs [150]. Sox17 could therefore be a distinguishing factor for fetal HSCs in transcriptional regulation.

Mutations in the proto-oncogene c-myb and the tumor suppressor protein Ikaros (regulation through chromatin remodeling) also affect definitive hematopoiesis, but hardly alter YS hematopoiesis [151, 152]. HSCs are migratory by nature which is evident from their migratory behavior in the embryo before homing to the adult BM niche before birth. Genes that are involved in HSC migration or homing such as integrins can therefore also affect hematopoiesis. As hematopoietic cells no longer differentiate if the homing or migration to the FL is compromised, this indicates that the P-Sp/AGM is incapable to sustain hematopoiesis.

Extra- and intraembryonic HSCs are ultimately derived from the same combination of mesoderm and endoderm layers (the Sp); mesoderm and visceral endoderm form the YS, and mesoderm tied to definitive endoderm in the AGM. Initially they share a c-KIT+/CD31+/CD41+/CD45- phenotype, but even though the early development might seem similar, only the P-Sp/AGM region ultimately produces definitive HSCs at first. In each compartment, it's likely that the sequence of events is identical and that they give rise to the same type of hematopoietic precursor, but that the environment determines the development of the involved cell types. In an experiment in *Xenopus* where the intra- and extraembryonic compartments were exchanged (reciprocal grafts) the regions are initially bipotent in primitive and definitive hematopoiesis, until commitment takes place during the neurula stages [153]. This indicates that the environment during the neurula stage rather than the tissue of origin plays a key role in the commitment of hematopoietic progenitors. This explains why some genes can affect primitive hematopoiesis, definitive hematopoiesis or both. Additionally, progenitors from the early yolk sac gain LTR activity when exposed to an AGM-derived cell line which implies that the YS environment constraints the development of definitive HSCs [154]. Forced HoxB4 expression can also induce early YS progenitors to an definitive HSC phenotype with LTR activity [155]. These results stress the importance of research on the environmental factors involved in the differentiation and self-renewal of hematopoietic progenitors.

2.7 Signaling Pathways and Exogenous Growth Factors

The TGF-β family member BMP4 plays a crucial role in hematopoiesis. Once activated it regulates the transcription of target genes such as Runx1 and GATA2 [156, 157]. In both human and mouse, BMP-4 is expressed in the mesenchyme underlying the ventral wall of the dorsal aorta. [156, 158, 159]. BMP-4 is not only involved in the induction of YS hematopoiesis, but plays a role in the maintenance of HSCs in the AGM by increasing their growth and/or survival [159, 160]. It was also demonstrated with the embryonic stem (ES) cell differentiation system that BMP-4 is required for the production of hematopoietic cells [161, 162]. Also, the self-renewal of cord blood derived human immature hematopoietic progenitors is dependent on BMP-4 [163].

Not surprisingly the developmental signaling pathways HH, Notch and Wnt play a role in hematopoiesis, though this is likely reflecting a general role in development and some results appear contradictory similar to result in the adult BM (see review [164]). In the embryo, Notch is required for GATA-2 expression in the AGM and the formation of intra-embryonic hematopoietic cells, while the HH pathway regulates primitive hematopoietic cells [18, 165, 166].In general, these pathways are involved separately or in combination (including BMP) in cell specification and hematopoiesis in the embryo.

Little is known about the soluble factors involved in HSC regulation in the embryo [167, 168]. Oncostatin M (OSM) was shown to induce differentiation of endothelial and hematopoietic cells in combination with SCF and basic fibroblast growth factor (bFGF) *in vitro* [169]. However, knockout OSM receptor mice only display defects in the erythroid and megakaryocyte lineages [170]. Additionally, OSM affects HSCs indirectly through gp130 which is required for the expansion of immature AGM hematopoietic progenitors [171].

TGFβ1 and macrophage colony stimulating factor (M-CSF) are known to influence hematopoietic development and VEGF and its receptor Flk1 (VEGF-R2) affect the development and/or migration of specifically hematopoietic and endothelial cells [167, 168, 172]. Flk1 knockout mice lack all cells from these lineages and VEGF is a major factor involved in blood island development [173].

Prostaglandin E2 (PGE2) was first found in zebrafish to play a key role in the self-renewal of HSCs and could expand HSC number 2 to 4-fold [174]. It is required for the Wnt activation in HSCs by degrading β-catenin [175]. Factors involved in the synthesis of PGE2 are also required for the formation of HSCs. Interestingly they found that PGE2 could improve the recovery of kidney marrow in irradiated zebrafish adults. Similar results were obtained in mice where exposure of embryonic stem cells to PGE2 amplified the number of multipotent progenitors and increased the frequency of LT-HSCs in the

BM after transplantation. Recent work has demonstrated that human HSCs also express PGE2 receptors and that PGE2 enhances the survival and homing to the BM niche of HSCs [176, 177].

3. Embryonic Stem Cells and induced Pluripotent Stem Cells

ESCs are pluripotent, meaning they can differentiate into derivates of all three germ layers. During normal development, the embryo is formed from the inner cell mass (ICM) of the blastocyst, while the outer layer becomes the extra-embryonic membranes and the placenta. When cultured *in vitro*, cells of the ICM give rise to embryonic stem cells which can be stimulated to differentiate into a wide variety of cell types including nerve, pancreatic, bone and cardiac cells.

In the 1960, several labs independently demonstrated that a single cell taken from a teratocarcinoma, an embryonic carcinoma (EC) cell, could be grown in culture as a stem cell [178]. Though initially used as an *in vitro* model for mouse early development, EC cells often have an abnormal karyotype and genetic mutations that make them less suitable to study normal development. Further investigation revealed that they strongly resemble stem cell behavior and are derived from a germ line stem cell. Twenty years later, two independent labs were able to derive normal ESCs from the inner cell mass of mouse embryos [179, 180]. In 1998, the group of James Thomson was the first to develop a technique to maintain and expand human ES cells in cell culture and ESCs are currently widely used in research [181].

Recently, the groundbreaking work of Dr. Yamanaka demonstrated that differentiated adult somatic cells could be reprogrammed into embryonic state stem cells, called iPSCs, through ectopic expression of a defined set of genes [182-184]. ESCs and iPSCs are strikingly similar in their morphological, molecular, and functional characteristics. Most notably both cell types can develop teratomas and differentiate into cells of all three primordial germ layers [181, 184]. Several adult cell types including keratinocytes, hematopoietic cells and (mostly) dermal fibroblasts can now be reprogrammed into iPSCs [185-187].

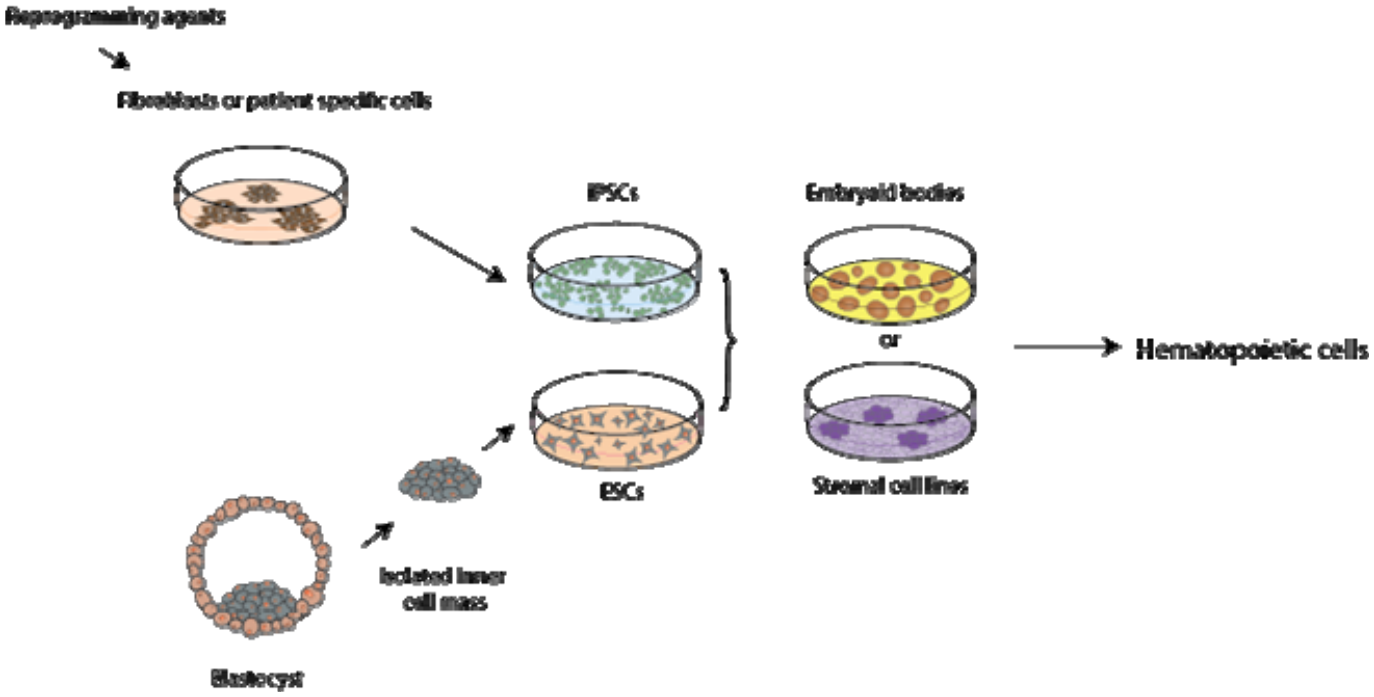


Figure 5. The generation of HSCs from ESCs and iPSCs

Embryonic stem cells (ESCs) can be cultured *in vitro* through isolation of cells from the inner cell mass. Induced pluripotent stem cells (iPSCs) are generated from differentiated cells with reprogramming agents such as proteins or genetic vectors. ESCs and iPSCs can subsequently produce hematopoietic cells, including HSCs, by embryoid body formation or with stromal cell lines.

Current progress has facilitated the generation of iPSCs without viral or vector integration and the prospect of using ESCs and iPSCs in the clinic are becoming more interesting and more feasible. [188-191].

One promising application of ESCs and iPSCs in the clinic is the generation of hematopoietic cells and study of hematopoiesis [192]. They can be used to study various processes such as human developmental biology, genetics and gene therapy, and provide a source for cell transfusions, immune therapies and transplantations. Especially iPSCs could be a suitable source for hematopoietic cells as they would probably lack an immunological response in patients after transplantation.

Doetschman *et al.* were the first to show that murine ESCs can develop into blood islands through embryoid body (EB)-mediated differentiation [193]. Lateron, the group of Dan Kaufman generated the first hematopoietic cells, including erythroid cells, from human ESCs derived hematopoietic precursor cells [194]. The derivation of erythrocytes from ESCs could provide a safer alternative to the current blood transfusions that are dependent on donors for blood. It has been demonstrated that erythrocytes can be produced from ESCs, but it remains difficult to obtain large enough numbers of

erythrocytes from ESCs or iPSCs for clinical use [195].

Differentiation of hematopoietic cells from ESCs can be done by either EB-mediated differentiation or by co-culturing of ESCs with stromal cell lines including AGM and FL-derived monolayers [194, 196-199]. The OP9 stromal cell line, deficient in M-CSF production, is most often used to study hematopoiesis from ESCs. This co-culture system has been used to generate megakaryocytes with a similar function and morphology to normal megakaryocytes [200]. Interestingly, normal embryonic development seems to be reflected in ESCs hematopoietic differentiation. After the formation of hemangioblasts, primitive erythroid progenitors and other progenitors arise that strongly resemble cells from yolk-sac hematopoiesis. Definitive hematopoiesis, which is characterized by lymphoid cells, can be induced in ESC cultures grown on OP9 stromal cell lines [201, 202]. Though immune cells such as T and B cells, NK cells and other immune cells for immune therapies can currently be formed from ESCs, several issues with the use of ESC or iPSC-derived hematopoietic cells will need to be addressed before application in the clinic. These issues include normal biological function (quality), quantity, safety (teratomas), maturity (adult versus embryonic developmental characteristics) and immune responses of the produced cells. Nevertheless, the first clinical trial with ES cell derived oligodendrocytes for the treatment of spinal cord injuries started early 2009 and two others clinical trials with ESC-derived retinal pigment epithelial cells and pancreatic progenitor cells have started since then [203]. Once the fore mentioned issues can be overcome, the use of ESC and iPSCs will greatly benefit patients around the world.

Though all mature hematopoietic cell lineages could be formed from ESCs or iPSCs, a straightforward goal that has yet to be achieved, is the isolation of putative HSCs with LTR capacity. Despite extensive efforts the development of *bona fide* HSCs from ESCs remains difficult. Daley and colleagues first demonstrated that forced expression of HoxB4 in mouse ESCs grown with an OP9 stromal cell line, enables the development of long-term engrafting primitive hematopoietic cells which can reconstitute both primary and secondary irradiated recipients [155]. HoxB4 target genes include regulators of the Wnt, Notch, and BMP signaling pathways [204]. Overexpression of the LIN homebox transcription factor Lhx2 could also provide reconstitution with ESC and iPSC-derived HSCs in irradiated mice, though much less efficient (only 4 months) [205].

In another approach, transplantable cells from ESCs were generated in culture with methylcellulose, stem cell factor, IL3 and IL6, but this approach is not widely reproducible due to the use of serum [206]. Several other groups have also demonstrated long-term engraftment of ESC-derived hematopoietic cells [198, 207-209]. Ledran and collegues show that co-culture of ESCs with specifically AGM derived stromal cell lines, led to higher levels of hematopoietic engraftment in severe immunodeficient mice

[198]. Human iPSCs have also been show to differentiate into hematopoietic cells, but with lower efficiency [192, 210]. Runx-1 is also important for the induction of HSCs from ESC in combination with IL-3 and OSM [159]. Runx1+/- mice display a low hematopoietic activity which could be rescued by the injection of IL3 in both the YS and placenta. *In vitro* experiments show that Runx1+/- cells can solely provide long term reconstitution in the presence of IL-3 and OSM.

Nevertheless, overall engraftment levels in experiments thus far are still far from optimal and appear to be restricted to the myeloid lineages. Moreover, ESC-derived hematopoietic cells seem embryonic in nature while definitive or mature hematopoietic cells including HSC are preferred. Elucidating the pathways and factors involved in definitive hematopoiesis seems inevitable to eventually generate *bona fide* HSCs from ESC.

Discussion

Hematopoietic stem cells give rise to all bloods cells and are the key factor in bone marrow transplantations to restore the blood system of patients. HSC could also provide a therapeutic source for a wide variety of diseases such as immune disorders. Currently, our only sources of transplantable HSCs are the bone marrow and umbilical cord blood which are limited in number of HSCs. As current isolation procedures for HSCs from the bone marrow are also far from efficient, there is a demand for ways to generate larger numbers of HSC either through expansion or formation from embryonic stem cells or induced pluripotent stem cells. The generation of HSCs from ESCs or iPSCs seems promising, though primarily primitive HSCs are produced. Embryonic primitive HSCs are first found in the embryonic yolk sac and cannot sustain adult hematopoiesis. Only definitive HSCs are capable of long-term reconstitution of the blood system in irradiated adults. Adult definitive HSCs originate in the AGM and can later be detected in the major arteries and placenta. The onset of circulation allows HSCs to migrate within the embryo and around midgestation they colonize the FL and eventually seed the bone marrow shortly before birth.
To understand the difference between the formation of primitive and definitive HSCs, we need to determine the factors involved in their development such as signaling pathways, extracellular factors and the role of the niche. Identification of important genes or proteins could allow the efficient production of definitive HSCs from ESCs or iPSCs or efficient expansion from existing sources *in vitro*.

Recently, an interesting experiment has shown that exocrine pancreatic cells can be converted into endocrine pancreatic cells without an iPSC intermediary [211]. It seems likely that trans-differentiation of somatic cells will be possible, mostly in similar cell types. Therefore, it could be possible to generate HSCs through trans-differentiation with defined factors. In that regard, the group of Mick Bhatia produced hematopoietic cells from OCT4 expressing fibroblasts [212]. Though cells from the lymphoid lineage were not detected, the CD45+ cells express adult globins indicative of definitive hematopoiesis. This raises the interesting opportunity to generate HSCs from somatic cells without traversing a pluripotent state.

Bone marrow transplantations have been applied in the clinic with much success for many years. New developments even allow the extraction of (low numbers of) HSCs from umbilical cord blood. Nevertheless, the use of HSCs remains limited due to limited expansion *ex vivo* and low engraftment levels. To date, activation of Notch and overexpression of HOXB4 or SALL4 have shown to be potent expansion factors of HSCs. In a clinical trial, it was demonstrated that Notch activation in umbilical cord blood cells can expand HSCs numbers 160-fold [213]. However, the expansion was predominantly

among short reconstituting hematopoietic cells.

The HOXB4 protein is an expansion factor of mostly mouse, but also human primitive hematopoietic cells [214, 215]. The recent discovery that ectopic expression of HoxB4 in murine ES cells can generate and expand HSC that display long term reconstitution in mice, has gained a lot of attention [155]. Wang *et al*. demonstrated that ectopic expression of HoxB4 in human ESCs increased the number of derived HSCs, but did not improve their reconstitution ability or function [216]. Additionally, they show that human ESC-derived HSCs could only engraft when injected directly into the BM, not through intravenous delivery. This observation was also documented with murine ESC-derived hematopoietic cells [206]. HoxB4 expression generates HSCs with unique behavioral properties (proliferation, migration) from somatic HSCs [216]. Schiedlmeier *et al*. showed that differentiating ESCs regulate genes more selectively upon HoxB4 expression than adult HSCs [204]. This demonstrates that there are still marked differences between somatic HSCs and HSCs derived from ESCs through HOXB4 expression. Though they might seem very similar, *in vivo* they can function very differently. Therefore, it will be important to compare somatic HSCs and ESC-derived HSCs molecularly.

One very interesting candidate, SALL4, has very recently come forward as a master regulator in the expansion of HSCs with long-term reconstitution ability. The zinc-finger transcription factor SALL4 is known to regulate the expression of Oct4 and Nanog and maintain ESC pluripotency [217]. Additionally, SALL4 is important for the self-renewal of both ESCs and HSCs, as well as the expansion of HSCs [218]. The number of CD34+ human HSCs can be expanded by more than a 10.000-fold by exposure to a TAT-SALL4 fusion protein and cytokines *ex vivo*. More importantly, the human HSCs display enhanced engraftment and long-term repopulation capacity in immunodeficient mice. The expanded cells display normal behavior in the bone marrow niche and there was no evidence of leukemia. This is the first experiment that has shown that long-term reconstituting HSCs can be amplified, engraft efficiently and seem safe to use in the clinic. Nevertheless, though results look very promising they will need to be confirmed by other labs and in nonhuman primates, followed by clinical trials to further review the safety of SALL4 expanded HSCs.

ESCs and iPSCs offer interesting ways to generate (patient-specific) stem cells. However, our understanding of reprogramming and the development of HSCs from iPSCs and ESCs is still not complete and many factors such as signaling pathways and transcription factors can affect the transcriptional state of a cell. It seems likely that the number of factors used for reprogramming in the future will increase to generate more specific iPSCs. The same will also apply for the generation of HSCs from ESCs or iPSCs. It is likely that a combination of factors will be required, in contrast to the expression of a single gene, to confer a hematopoietic identity.

The directed differentiation of hematopoietic cells from ESCs or iPSCs can either be done by embryoid body formation in suspension culture or with through co-culture with stromal cell lines (OP9). Currently ESC-derived hematopoietic cells are primarily primitive, which give rise to progeny that express embryonic globins and lack lymphoid potential. Hence, it will be important to identify the molecular program that can induce a shift from HSCs with primitive to definitive hematopoietic potency.

Even with the success of ESC-derived HSCs through HOXB4 or SALL4 expression, it remains a challenge to generate expandable, engraftable, transplantable, definitive HSCs capable of multi-lineage (especially lymphoid) long-term reconstitution. In conclusion, improvements in both reprogramming and differentiation will have to be made to apply ESC or iPSC-derived HSCs successfully in the clinic.

List of abbreviations

Below is a list of used abbreviations in alphabetical order.

AGM	Aorta-Gonad-Mesonephros
bFGF	Basic Fibroblast Growth Factor
BM	Bone Marrow
BMP	Bone Morphogenetic Protein
CFU	Colony Forming Unit
EC	Embryonic Carcinoma
ESC	Embryonic Stem Cell
FL	Fetal Liver
GFP	Green Fluorescent Protein
HH	HedgeHog
HLA	Human Leukocyte Antigen
HSC	Hematopoietic Stem Cell
IAHC	Intra-Aortic Hematopoietic Cluster
ICM	Inner Cell Mass
iPSC	Induced Pluripotent Stem Cell
LDL	Low-Density Lipoproteins
LSK	Lin-/Sca1+/c-KIT^{hi}
LT	Long Term (reconstituting)
LTR	Long Term Reconstitution
M-CSF	Macrophage Colony Stimulating Factor
NK	Natural Killer
OSM	Oncostatin M
PGE2	ProstaGlandin E2

Pl	Placenta
P-Sp	Para-aortic Splanchnopleura
SAP	Sub-Aortic Patches
SCF	Stem Cell Factor
ST	Short Term (reconstituting)
STR	Short Term Reconstitution
TGF-β	Transforming Growth Factor Beta
UA	Umbilical Arteries
VA	Vitelline Arteries
VEGF	Vascular Endothelial Growth Factor
YS	Yolk Sac

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